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(54) Title: PROCESS OF OBTAINING COLLOIDAL DISPERSIONS OF AMPHOTERCIN B; COMPOUNDS BASED ON AMPHOTHERICINE B FOR ENTERAL, PARAENTERAL AND TOPICAL USE; USE OF THESE COMPOUNDS IN THE TREATMENT OF SYSTEMIC MYCOSES AND PARASITIC INFECTIONS

(57) Abstract: The present invention has as its objective the process of obtaining of compositions containing amphotericin B in its superaggregate from capable of treating infections caused by fungi, parasites and other agents susceptible to this antibiotic. The invention covers the stable formulation of amphotericin B in its superaggregate form in presence of sodium deoxycholate, freeze-dried (lyophilized) and sterile, uniting the requirements for an injectable formulation for use enteral, parenteral or topical, with reduced toxicity in comparison to the conventional forms that transmit amphotericin B.

DESCRIPTIVE REPORT OF THE PATENT OF INVENTION FOR "PROCESS OF OBTAINING COLLOIDAL DISPERSIONS OF AMPHOTERICIN B; COMPOUNDS BASED ON AMPHOTHERICINE B FOR ENTERAL, PARAENTERAL AND TOPICAL USE; USE OF THESE COMPOUNDS IN THE TREATMENT OF SYSTEMIC MYCOSES ANDF PARASITIC INFECTIONS."

The present invention refers to the process of obtaining pharmaceutical compounds consisting of colloidal dispersions of amphotericin B obtained by the heating of the antibiotic in the presence of biliar salts, resulting in stable pharmaceutical compounds, which could be freeze-dried (lyophilized) and enable to be reconstituted in water-based solutions usable paraenternal, or in a gel form. It refers also to its use in the treatment of infections whose etiological agents are susceptible to this antibiotic after administration intestinally, paraenteral and topically.

Amphotericin B (AMB) is a polyene antibiotic, with a wide spectrum of antifungal action, isolated in 1953 from a culture of *Streptomyces nodosus*. It is understood as a macrolides that contains, on one side of the lactone ring, 7 trans conjugated double bonds and one 3-amino-3, 6-didesoxymannose (micosamine) group connected to the ring by a glycosidic bond; and on the other side an equal number of hydroxylic groups.

The structure of AMB is highly lipophilic and not really dissoluble in water. Due to the presence of the carboxylic group on the main ring and the NH_2 group of micosamine the AMB possesses an amphoteric behaviour, forming soluble salts at extreme pHs. Despite being insoluble in water the AMB is commonly administered intravenously in a formulation that contains biliar salt sodium deoxycholate (DOC) as a tensoactive (Barner et al., Antibiotics Annual 1957-1958: pp. 53-58) commercially known as Fungizon® and owned by Bristol Myers Squib.

The antifungal activity of AMB is due to its affinity for the sterols of the cell wall of the susceptible fungi. The link of AMB to ergosterol results in disruption of the cellular membrane, possibly by the formation of pores consisting of AMB joined with sterol. These effects cause the depolarization of the membrane and the increase in permeability to protons and monovalent

cations, which permits the loss of the cytoplasmic contents bringing about the death of the cell (Brajtburg et al. Amphotericin B: Current understanding mechanisms of action. Antimicrobials Agents Chemotheraphy, 34(2): 183-88, 1990.). However, AMB's action on fungal cells involves more than one mechanism. It has been shown that the lethal effects of high concentrations of AMB on *Candida albicans* are not simply a consequence of an alteration created in the function of the membrane barrier, but involve oxidative damage. This pharmaceutic seems to cause a cascade of oxidative reactions starting with its own oxidation (Sokol-Anderson ML, Brajtburg J, Medoff G. Amphotericin B-induced oxidative damage and killing of *Candida albicans*. Journal of Infectious Diseases, 154:76-83, 1986). Besides this, AMB has immunomodulating effects on several types of cells. AMB increases the response of macrophages against various pathogens and causes these cells to increase production of TNF-α, NO and types reactive to oxygen. (Mozaffarian et al. Enhancement of nitric oxide synthesis by macrcphages represents an additional mechanism of action for amphotericin B. Antimicrobial Agents and Chemotheraphy 41(8): 1825-29, 1997).

AMB is the most powerful antifungal available, being the drug of choice for the treatment of systemic mycoses, especially in patients with a compromised immunological system. Clinical studies currently in progress show the interest of this antibiotic against different manifestations of leishmaniasis. (Yardley and Croft. A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. International Journal of Antimicrobials Agents, 13(4):243-248). Nevertheless, the use of this pharmaceutical is limited by the sharp and chronic reactions following its administration. Nephrotoxicity is the severest adverse chronic effect associated with AMB and could be classified as tubular and glomerular. The clinical and laboratorial manifestations include lowering the rate of glomerular filtration and the flow of renal blood, hypocalaemia, hypomagnesemia, tubular acidosis and nephrocalcinosis.

The distribution of this pharmaceutical, its clinical effectiveness and toxicity, are all related to the liberation of the AMB of the formulation and its

availability to the affected cells. The bioavailability of AMB is also moderated by its high capacity to link lipoproteins. In the blood, 90 to 95% of the AMB is mainly associated with LDL (Petit et al. In vivo therapeutic efficacy in experimental murine mycoses of a new formulation of deoxycholate-amphotericin B obtained by mild heating. Journal of Antimicrobial Chemotherapy, 42: 779-85, 1998). One of the mechanisms involved in the chronic toxicity of AMB is its endocitosis mediated for LDL by specific receptors, bringing the pharmaceutical to high intracellular concentrations, which results in the inhibition of endosomic and lysosomic fusion (Vertut-Doi et al. The endocytic process in CHO cells, a toxic pathway of the polyene antibiotic amphotericin B. Antimicrobials Agents and Chemotherapy. 38(10):2373-79, 1994). Some research suggests that elevations in the levels of LDL are associated with the increase in renal toxicity caused by AMB (Wasan et al. Influence of lipoproteins on renal cytotoxicity and antifungal activity of amphotericin B. Antimicrobials Agents and Chemotherapy. 38(2):223-27, 1994).

In general, the intravenous infusion of AMB-DOC is badly tolerated, and associated with the above-mentioned side-effects and with high mortality, requiring, in some cases, interruption in treatment (Saxena S, Khan JA, Ghosh PC. Toxicity and therapeutic efficacy of amphtericin B delivered through hemisccinate vesicles in the treatment of experimental murine aspergillosis. Antimicrobial Chemotherapy. 42(5):635-42, 1998). Added to this fact the high incidence and severity of opportunistic fungal infections and the growing number of pathogens resistant to the pharmaceuticals available, it is apparent that the therapeutic efficiency of anti-fungal treatments must be improved (Clemons K.V. and Stevens D.A. Comparison of Fungizone, Amphotec, liposome and Abelcet for treatment of systemic murine cryptococosis. Antimicrobials Agents and Chemotherapy. 42(4):899-02, 1998).

Its chemical nature and capacity for integration in biological membranes makes AMB an appropriate substance to incorporate in lipidic systems, like, for example, liposomes. Lipidic formulations have been widely explored as carriers of pharmaceuticals. (Szoka et al., Antimicrobial Agents and Chemotherapy 31:421-429, 1987; Adler-Moor et al., US Patent 5.043,107; Lopez-Berestein et

al. Treatment and prophylaxis of disseminated infection due to Candida albicans in mice with liposome-encapsulated amphotericin. Journal of Infectious Disease, 147(5):939-44, 1983; Mehta et al. Liposomal associated amphotericin B is toxic to fungal cells but not to mammalian cells. Biochimica et Biophysica Acta, 770:230-34, 1984). These lipidic formulations alter AMB's pharmacokinetic properties and its pattern of distribution in tissues. Currently, three lipidic formulations containing AMB are being clinically evaluated: Amphotec (ABCD); Sequus Pharmaceuticals, Inc), Ambisome (AMB-L; Nextar Pharmaceuticals, Inc) and Abelcet (ABLC, Lipossome Co., Inc). These three products differ in the types and proportions of phospholipids: AMB, which can be an important determinant of the antifungal activity and of the toxicity. The pharmacokinetic, tecidual and plasmatic properties are also different in the three formulations. However, the clinical significance of these differences has not been established (Wong - et al. Lipid formulation of amphotericin B: clinical efficacy and toxicities. Journal of Infectious Disease 27:603-18, 1998). Differences and in potency and therapeutic effectiveness exist among these lipidic systems, however, all are less toxic than Fungizon®, allowing the administration of superior doses, producing better therapeutic results (Johnson et al. Comparison of in vitro antifungal activities of free and liposome encapsulated nystatin with those of four amphotericin B formulations. Antimicrobials Agents and Chemotherapy. 42(6):1412-16, 1998.; Clemons K.V. and Stevens D.A. Comparison of Fungizone, Amphotec, liposome and Abelcet for treatment of systemic murine cryptococosis. Antimicrobials Agents and Chemotherapy. 42 (4) :899-02, 1998). Several mechanisms can participate in the reduction of the toxicity of the lipidic formulations of AMB. According to Mehta and collaborators, the improvement in the therapeutic index observed with the administration of AMB-Lip in relation to free AMB is fundamentally due to the alteration of the capacity of the drug to interact with the membranes containing cholesterol. The liposomes can selectively transfer AMB to the fungal cells, avoiding mammal cell membranes (Mehta et al. Liposomal associated amphotericin B is toxic to fungal cells but not to mammalian cells. Biochimica et Biophysica Acta, 770:230-34, 1984).

The pharmacokinetic alterations of AMB, or its distribution AMB can also contribute to reduction of the toxicity. According to the literature, the liposomes of AMB provide a reduction of the renal concentration of AMB and elevation of the concentrations in the organs of the mononuclear phagocytic system (SFM), mainly the liver and spleen (Boswell et al.Toxicological profile and pharmacokinetics of a unilamellar liposomal vesicle formulation of amphotericin B in rats. Antimicrobials Agents and Chemotherapy. 42(2):263-8, 1998.; Profitt et al. Pharmacology and toxicology of lipossomal formulation of amphotericin B (AmBisome) in rodents. Journal of Antimicrobials and Chemotherapy 28(Supp1. B) :4961, 1991.). A third possible mechanism is the selective degradation of the liposomes for phospholipases and lipases present in the fungi liberating the pharmaceutical directly into the target cells (Perkins et al. Amphotericin B-phospholipid interactions responsible for reduced mammalian cell toxicity. Biochimica et Biophysica Acta. 1107:271-82, 1992; Hutchaleelaha et al. Comparative pharmacokinetics and interspecies scaling of amphotericin B in several mammalian species. Journal of Pharmacy and Pharmacology 49:178-83, 1997).

In aqueous solution AMB exists as a mixture of different types in balance monomers soluble oligomers and insoluble aggregates. The proportion of each form depends on the concentration and the solvent of the stock solution (Legrand et al. Effects of aggregation and solvent on the toxicity of amphotericin B to human erythrocytes. Antimicrobials Agents and Chemotherapy. 36(11):2518-22, 1992). The activity and the selectivity of AMB demonstrate correlation to the aggregation state and size of the particles. In accordance with Glues and Dussault, the toxicity of this antibiotic administered as monômeros is decreased (Gruda & Dussault. Effect of the aggregation state of amphotericin B on its interaction with ergosterol. Biochemical Cell Biology. 66(3)177-83, 1987). Legrand and collaborators (1992) concluded that the form of AMB that promotes cytotoxic events in human erythrocytes is the aggregate water-soluable form. Among the strategies to reduce the toxicity of AMB, the development of new derivations and/or formulations that cause a decrease in the amount of aggregation or that originate less toxic aggregates has been

receiving attention. It was demonstrated by Gaboriau and collaborators solutions of AMB-DOC obtained from a starting point of the commercially available formulation (Fungizon®), when heated, induce the formation of superaggregates of this antibiotic. These changes in the aggregation state result from the condensation of the monomérica and aggregate forms, establishing a new equilibrium. It was observed that the heated solutions of AMB-DOC showed a significant decrease of the toxic effects, while maintaining their antifungal activity. The low toxicity of this formulation probably results from the different physiochemical properties of the superaggregated state and from the distribution pattern (Gaboriau et al. Heat-Induced superagregation of anphotericin B reduces its in vitro toxicity: a new way to improve its therapeutic index. Antimicrobials Agents and Chemotherapy. 41 (11) :2345-51, 1998. Petit et al. In vivo therapeutic efficacy in experimental murine mycoses of a 20 new formulation of deoxycholate-anfotericin B obtained by mild heating. J Antimicrob Chemother. 42: 779-85, 1998).

Some studies indicate that the ligation of AMB free to LDL is reduced when it is administered in the heated form in comparison with the conventional preparation, which would reduce the endocytosis of this pharmaceutical mediated by receptors specific for LDL. Independent of the process of endocytosis mediated by specific receptors, the larger size of the superaggregates of heated solutions (0.6 µm) allows these to be captured with greater efficiency by the cells of the (SFM) than by the small aggregates of the unheated formulation. In this way, the administration of AMB-DOC in the superaggregate form could generate higher concentrations in tissues for longer periods of time. This formulation would have similar properties to the liposomes of AMB, that is, it would act as a reservoir of the monomeric form, reducing the toxicity of AMB. The auto-oxidation of AMB in solution, as well as the induction of the lipidic peroxidation of the graxos unsaturated acids of the cellular membrane, are promoted by the type reactive to oxygen that can be produced by this antibiotic. Thus, the toxic events related with the oxidative process would also be minimized in the superaggregate form due to its greater stability in relation to the conventional form (Legrand et al. Effects of aggregation and

solvent on the toxicity of amphotericin B to human erythrocytes. Antimicrobials Agents and Chemotherapy. 36(11):2518-22, 1992). These data suggest that the heating of the solutions of AMB-DOC, to 70°C for 20 minutes, can be a simple way of improving the therapeutic index of this drug.

However, the studies accomplished up to now are based on the re-suspension of the freeze-dried (lyophilized) formulation commercially available in an aqueous solvent, followed by the heating. This formulation should be used quickly due to problems of stability. The problems related to this process would be mainly linked to the difficulties of application of the medication, in the hospital environment, because it would be necessary for qualified personnel to previously prepare the formulation for the administration of the medicine, since the supreaggregate form is not available ready for use. In other words, at the present time, there no process that prescribes a method of obtaining the formulation of amphotericin B in its superaggregate state that would result in a freeze-dried (lyophilized) formulation, stable and ready to be injected.

Fungizon® is marketed as a freeze-dried (lyophilized) powder that contains 50 mg of AMB, 41 mg of DOC and 20 mg of phosphate cap, as a compound AMB-DOC. When in solution AMB DOC forms a colloidal dispersion with particles measuring about 0.4 µm in diameter. After its administration, this formulation is dissociated into DOC and free AMB. Seeking to improve the therapeutic index of AMB and to reduce the incidence of adverse reactions related to its administration, new derivations and formulations of AMB have been proposed.

Proposals include patents US 4.522.803; US 4.963.297 and US 5.616.334, which describe the obtaining of amphotericin B compositions consisting of liposomes or complex lipidics for paraenteral administration. As previously mentioned, the lipidic compositions present a variety of inconveniences, from the complexity of their preparations to the high cost involved.

The patent US 5.776.904 describes a process of obtaining amphotericin B dispersions, without assistance, designed for paraenteral administration,

however, this patent describes the process of obtaining the dispersion through expensive equipment that require a significant consumption of energy.

Through the references cited, it is possible to identify a great variety of compositions found in the development and/or marketing of AMB. However, none of those compositions combines the properties of effectiveness, low cost and technical ease in their production. Although these formulations containing AMB have been proven to be well tolerated by the patients, reducing the incidence of sharp adverse reactions and of nephrotoxicity, the process of obtaining these presents high cost and difficulties. Thus, none of these products should be considered as standard therapy for systemic mycoses and much less for tropical diseases such as leishmaniasis; its usage being presently limited to patients who demonstrate little tolerance for, or who don't respond to the conventional treatment with AMB-DOC.

Previously it was mentioned that the compositions of AMB in the superagreggate form present the capacity to reduce the toxicity of the amphotericine B while maintaining its activity. In spite of this formulation presenting several therapeutic advantages, the technique involved in the obtaining of these superaggregates is ineffective from the point of view of stability of the formulations; since after the reconstituting and heating, the preparations must be used quickly. It is also a homegrown technique, involving the preparation of the compositions at the moment of their use, a fact which impedes its application in a simple and widespread way by medical professionals, due to the impossibility of analytical control of these compositions when being prepared.

One of the objectives of the present invention is the process of obtaining compositions containing amphotericin B in its superaggregate form, without the use of equipments that require high energy consumption, capable of treating infections caused by fungi, parasites and other agents affected by that antibiotic.

The obtaining of the formulations for parenteral and topical use containing amphotericin B in its superaggregate form, following the procedure of the invention herein, can be achieved using the following protocols:

Protocol A:

(i) Dissolve a biliar salt, preferably sodium deoxycholate in water to obtain a solution of final concentration varying from 0.001M to 1, OM;

(ii) Add to the solution (i) the amphotericin B, in the amount sufficient to obtain a final concentration of amphotericin B in solution (i) varying from 0.0005M the 0.5M and maintain the mixture under agitation;

(iii) Cool the mixture obtained in (ii) to a temperature between 1°C and 10°C;

(iv) Alkalinize the dispersion adjusting its pH between 10 and 13;

(v) Neutralize the solution obtained in (iv) to a pH between 6 and 8;

(vi) Filter in sterilized filters of 0.22 µm;

(vii) Heat the solution to a temperature between 50°C and 90°C for 10 to 40 minutes under agitation;

(viii) Condition the product in appropriate containers and freeze-dry (lyophilize)it.

Protocol B:

(i) Dissolve a biliar salt, preferably sodium deoxycholate in water to obtain a solution of final concentration varying from 0.001M to 1, OM;

(ii) Add to the solution (i) the amphotericin B, in the amount sufficient to obtain a final concentration of amphotericin B in solution (i) varying from 0.0005M the 0.5M and maintain the mixture under agitation;

(iii) Cool the mixture obtained in (ii) to a temperature between 1°C and 10°C;

(iv) Alkalinize the dispersion adjusting its pH between 10 and 13;

(v) Neutralize the solution obtained in (iv) to a pH between 6 and 8;

(vi) Heat the solution to a temperature between 50°C and 90°C for 10 to 40 minutes under agitation;

(vii) Add a gelling agent in the aqueous phase, selected from among the derivatives of carboxymethylcellulose in concentrations varying from 1% to 6% in weight or hydroethylcellulose in concentrations varying between 0.5% to 5% or derived polymers of acrylic acid (carbopol) in concentrations of 0.5% to 5%, followed by neutralization with an alkaline agent;

(viii) Add a promoter of absorption from among the quaternary compositions of ammonium (preferably chloride of methylbenzethonium in concentrations from 5% to 15%), urea (5% to 10%) or polyols, preferably derivatives of cyclodextrine;

Additionally, the compositions prepared following Protocolo B can contain stabilizing agents so that they present a longer period of time before expiring. Among the stabilizers usually used in pharmaceutical practice the metasulfites of sodium or potassium in the concentrations of 0.05% to 0.2% are preferable, or chelating agents, such as tartaric acid or citric acid in concentrations from 0.01% to 1% in weight in the final composition.

In accord with to invention herein the amphotericin B used for the preparation of the superaggregates formulations can be used in its common crystalline usual form and/or in its amorphous form, this last one obtained by differentiated crystallization procedures as described by (Bartner et al., Studies on a new solubilized preparation of amphotericin B. Antibiotics Annual, 1957-1958: 53-58), where the amorphous form is prepared through the dissolution of the crystalline amphotericin B in dimethylsulfoxide, when the solution formed is added to a room temperature solution sodium chloride (0.4%) causing the amorphous precipitation of the amphotericin B.

Among the alkalinizing agents mentioned in the protocols described for the invention herein, basic inorganic salts can be used, such as the hydroxides of the alkaline metals, those of sodium and potassium, the salts of the alkaline earth metals, such as those of magnesium, calcium and barium, the carbonates or bicarbonates of the alkaline metals, such as those of sodium, potassium or of the alkaline earth metals, such as those of magnesium, calcium and barium. The alkalinizing agents listed could be used in their solid forms or in stock solutions in pre-defined concentrations, in a way that facilitates the adjustment of the pH of the prepared solutions.

The neutralization of the alkaline solutions can be made by the addition of appropriate inorganic acids such as hydrochloric, sulfuric or phosphoric acids, used in their concentrated forms or diluted, or by the addition of salts, such as the sodium or potassium phosphate, among others.

The prepared solutions according to the Protocol are processed in such a way that they will be obtained as sterile formulations, appropriate for injectable use. The sterilization process preferably adopted is of filtration through a sterilizing membrane of 0.22 µm. The non-sterilization of the product limits its use to parenteral or ophthalmic methods. However, in the formulations for topical use, the sterilization stage is not necessary, and conservative formulations could instead be added, preferentially esters of p-hydroxybenzoic acid (0.02% to 0.2%).

For the freeze-drying (lyophilization) of these solutions the methods usually used to obtain these types of formulations can be employed.

A second objective of the invention herein is to demonstrate the effectiveness of the prepared compositions in the gel form for topical use, whether containing stabilizers or not, in the treatment of infections caused by *Leishmania amazonensis*.

Leishmaniasis is an endemic disease in several areas of Brazil. Because of the fact of being a deforming affliction whose treatment is still extremely limited in terms of effective medicines capable reversing the unbridled damage, this disease is a very great reason for concern among the population subject to it as much as among the health professionals in search of an effective treatment.

A third objective of the invention herein is to facilitate the maintenance of activity of injectable compositions against *Candida albicans*, expandable to other fungal manifestations affected by the drug, and the maintenance of activity after heating under the described conditions, as well as reduction of the toxicity after intravenous administration.

The compositions for topical use in the gel form containing amphotericin B in the superaggregate form described in the invention herein presented the greatest activity in the combat against leishmaniasis. The studies in animals demonstrated these to be the most effective compositions in blocking the evolution of the disease and cicatrization of the lesions formed, when compared to the preparation of the gels with the conventional form of the amphotericin B.

The injectable compositions prepared in agreement with the present invention present a less toxic pattern than those prepared conventionally, including all those currently available on the market, such as, for instance, Fungizon® being appropriate for intravenous administration for the treatment of varied infections, particularly the infections caused by fungi and parasites.

These injectable freeze-dried (lyophilized) formulations, after redispersion in an aqueous solvent, that can be water, isotonic solution of sodium chloride (0,9%) or preferably isotonic solution of glucose (5%), which comes as a colloidal dispersion with particles that vary in diameter between 0.1 to 1.0 μm. The re-suspension of the formulations in an aqueous solvent happens spontaneously with a quick shake of the wrist without the need of mechanical agitation, and could be done by a health professional alone, immediately before administration of the drug, and without the need for technical personnel to aid in its preparation.

The compositions described in the present invention can be used in the treatment of various infections caused by fungi, parasites and/or other agents, being appropriate for use in humans and also in animals, and used in the fields of human and veterinary medicine.

The formulation obtained in the present invention possesses the advantage of not having in the composition lipids in the liposomal form or complexities that would result in higher costs in the production process and a decrease in the stability of the formulations.

A great advantage of the present invention consists in the reduction of the toxicity of the amphotericin B, since a differentiated bio-distribuition is observed among the two forms, resulting in the reduction of the toxicity when administered in the heated form.

The following examples are meant to be illustrative, not exhaustive; letting it be understood that the many variants for the present invention are not limited to these examples.

Example 1 – Process for obtaining the superaggregate form of amphotericin B

1) Dissolve 12.4 grams of sodium deoxycholate in 1320 ml of water for injectable form;

2) Add to this solution 15 grams of crystalline amphotericin B and let it agitate vigorously for one hour;

3) Cool to 7°C;

4) Adjust the pH to 12.0 adding 32.6 ml of sodium hydroxide (1N) with constant agitation until the dissolution is complete;

5) Adjust the pH from 7.0 to 7.6 with sodium phosphate

6) Heat to 70°C for 20 minutes;

7) Raise the volume to 1,500 ml;

8) Divide into fractional volumes of 5ml for freeze-drying (lyophilization).*

* The final formulation should be kept under refrigeration (at temperatures between 2 and 8°C).

Example 2 - Process for obtaining the superaggregate form of amphotericin B starting with the amphotericin in its amorphous form

1) Dissolve the correct amount of biliar salt, preferably sodium deoxycholate to obtain a molar proportion of 1:2 in relation to amphotericin B;

2) Add to this solution the amphotericin B in its amorphous form (Bartner et al., 1958) and let it agitate vigorously for two hours at room temperature;

3) Heat to temperatures between 50 to 80°C from 10 to 40 minutes;

4) Raise the volume by adding water until the desired concentration is reached;

5) Divide the solution into fractional volumes according to the desired concentration for liofilização.

6) Freeze-dry (lyophilize)

The formulation obtained in this way has the appearance of a freeze-dried (lyophilized) yellow powder. Total sterility of the formulation can be obtained by including in the process described above a step using a sterilizing filter. The final formulation should be kept under refrigeration (at temperatures between 2 and 8°C).

Example 3 - Process of obtaining the formulation of amphotericin B for topical use in its superaggregate form as a gel:

1) Dissolve 12.4 grams of sodium deoxycholate in 1320 ml of water for injectable use;

2) Add to this solution 15 grams of crystalline amphotericin B and let agitate vigorously for one hour;

3) Cool to 7°C;

4) Adjust the pH to 12.0 adding 32.6 ml of sodium hydroxide (1N) with constant agitation until dissolution is complete;

5) Adjust the pH to between 7.0 and 7.6 with sodium phosphate

6) Heat to 70°C for 20 minutes;

7) Raise the volume to 1,500 ml;

8) Add urea in a concentration of 10%

9) Add the hydroxyethylcellulose (natrosol) in a concentration from 1 to 2%;

10) Raise the volume by adding water until the desired concentration of amphotericin B is reached;

11) Store in places appropriate for the storage of semi-solid formulations.*

* The final formulation should be kept under refrigeration (in temperatures between 2 and 8°C).

Example 4 - Evaluation of the leishmanicidic activity of formulations containing AMB in its aggregate and conventional forms created for the topical treatment of cutaneous leishmaniasis in mice C57B16.

Infection of the mice: The mice C57B1/6 were infected with a sample of *Leishmania amazonensis* at the base of the tail. In general, this type produces, in humans, simple and limited lesions, containing numerous parasites at the edges of the lesions. Treatment was begun two months after the infection process showed the emergence of the nodules.

Treatment: The mice were treated topically with the gel formulations of conventional AMB and the heated form containing urea as a promoter of absorption. The treatment consisted of two daily applications at the site of the infection over a period of 30 days. The animals were organized into 3 groups of 11 mice each: 1) a control group not treated; 2) a group treated with heated AMB containing 10% urea topically applied; 3) a group treated with conventional AMB containing 10% urea, topically applied.

At the beginning of the treatment all the animals were found to be in the nodule stage that is characterized by intact epidermis, strongly infiltrated by microphages and numerous parasites.

The treatment was initiated after the lesions appeared, two months after infection. The mice were separated into groups (randomly) and treated according to the protocol described above.

In the control group, not treated, a rapid evolution of the illness was observed, that is the lesion spread in the animals. In the groups that received the topical treatment, the evolution of the disease was halted in 75% of the animals treated with the gel containing the promoter and AMB in the aggregate (heated) form. Their lesions scarred over. In the case of the gel containing the conventional form of amphotericin B, 63% of the animals had their lesions scarred over. Table 1 shows the evolution of the disease in the different groups.

Table I - Evolution of the lesions in mice infected with *L. amazonensis*.

Treatment	Days after beginning treatment				% scarred over
	17	36	51	58	
Heated AMB gel	++	++	++	+	75
Conventional AMB gel	++	+++	+++	++	63
Control Group (not treated)	+++	+++	++++	++++	0

Similar results were obtained in BALB/c mice infected with the same sample, showing no significant difference between concentrations of 0.25% and 1.0% of amphotericin B in heated form.

Example 5 - Evaluation of the activity against fungi

For the test of antimicrobiotic activity of heated amphotericin B obtained by the process of the present invention the dilution test was used on the amphotericin B in the conventional and heated forms in serial concentrations of half n° 3 containing a culture of *Cândida albicans* ATCC 2915. The smallest concentration of amphotericin B capable of inhibiting microbial growth after 18

hours of incubation (CIM – minimum inhibitory concentration) has been determined. The heating of amphotericin B solutions for 20 minutes to 70°C with the superaggregates formation does not cause a decrease in the activity and it could be proven with the determination of CIM that for 3 lots of amphotericin B in the conventional and heated form the same values were found for the two forms: 1.0µg/mL (Table II).

Table VI: Determination of the minimum inhibitory concentration (CIM) of 3 lots of AMB in the conventional and heated forms through the reading of the transmission percentage.

Formulations	Concentrations (mcg/mL)				
.	0,5	0,7	1,0	1,4	2,0
L1 Conv	96,5	97,7	100	100	100
L1 Heated	97,7	97,7	100	100	100
L2 Conv	96,6	98,8	100	100	100
L2 Heated	97,2	96,6	100	100	100
L3 Conv	97,5	98,0	100	100	100
L3 Heated	98,2	98,3	100	100	100

Example 6 - Evaluation of severe toxicity by the determination of the 50% lethal dose

The 50% (DL50) lethal dose of conventional and heated amphotericin B was determined in Swiss mice, using the intravenous administration. It was determined by the number of dead animals over a period of 15 days after administration of a single intravenous dose. Mice, weighing between 20-25 g, were separated into groups of 10 animals. The amphotericin B was administered into the vein of the tail in different doses (2 to 10 mg/kg). The injection was administered slowly for 30 seconds. The animals were examined in the same way in search of signs of intoxication, lethargy, modification of behavior and death. The 50% lethal dose was calculated at 4.5 mg/kg for the conventional formulation of amphotericin B. For the formulation in the superaggregate (heated) state a mortality was observed of only 20% of the animals in the dose of 10 mg/kg and 80% in the dose of 15 mg/kg. No animal

died with the dose of 5 mg/kg, corresponding to the closest dose to the 50% lethal dose for the unheated formulation.

CLAIMS

1 - Process of obtaining of colloidal dispersions of amphotericin B in the superaggregate form, ready for injectable use after dispersion in aqueous solvent and compositions based on superaggregate amphotericin B for topical use;

2 - Process of obtaining a pharmaceutical composition containing amphotericin B in the superaggregate form, without the presence of lipids, freeze-dried (lyophilized), stable and sterile, presenting inferior toxicity to the conventional form, ready for re-suspension in an aqueous solvent and characterized by the heating of this antibiotic in the presence of a biliar salt.

3 - Process of obtaining items 1 and 2 above, characterized by the fact of the amphotericin B being initially dispersed in an aqueous solvent in the presence of a biliar salt, preferably sodium deoxycholate, in alkaline pH that, soon afterwards is neutralized, and whose dispersion is submitted to heating in aseptic conditions before being freeze-dried (lyophilized) and contained.

4 – Process of obtaining items and processes referred to in 2 and 3 above, where the formulation of amphotericin B in its superaggregate state in presence of sodium deoxycholate is freeze-dried (lyophilized) and released in this pharmaceutical form, ready for re-constitution and administration.

5 - Process of obtaining items and processes referred to in 2, 3 and 4 above, characterized by a formulation of amphotericin B in its superaggregate state in the presence of sodium deoxycholate and freeze-dried (lyophilized) and dispensed in this pharmaceutical form, ready for re-constitution and administration to the patient, without the need of thermal treatment before administration.

6 - Pharmaceutical composition obtained by the process described in items 2, 3 and 4 above, ready for human or veterinary use, where the amphotericin B comes in the superaggregate state freeze-dried (lyophilized) with particles smaller than 1 μm.

7 - Pharmaceutical composition obtained by the process described in items 2, 3 and 4 above, that can be administered by the methods enteral, parenteral or topical.

8 - Pharmaceutical composition obtained by the process described in items 2, 3 and 4 above, compatible and appropriate for administration by the methods intravenous, intra-arterial, intra-spinal and subcutaneous.

9 - Pharmaceutical composition obtained by the process described in items 2, 3 and 4 above, where the amphotericin B in its superaggregate form in the presence of sodium deoxycholate can be dispensed in a gel for topical or injectable use.

10 - Pharmaceutical composition obtained by the process described in items 2, 3 and 4 above, where the amphotericin B in its superaggregate form in the presence of sodium deoxycholate can be dispensed in a gel with or without the presence of an absorption promoter.

11 - Pharmaceutical composition obtained by the process described in items 2, 3, 4, 9 and 10 above, that can be administered topically ophthalmically.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS OF OBTAINING COLLOIDAL DISPERSIONS OF AMPHOTERCIN B; COMPOUNDS BASED ON AMPHOTHERICINE B FOR ENTERAL, PARAENTERAL AND TOPICAL USE; USE OF THESE COMPOUNDS IN THE TREATMENT OF SYSTEMIC MYCOSES AND PARASITIC INFECTIONS

(57) Abstract: The present invention has as its objective the process of obtaining of compositions containing amphotericin B in its superaggregate from capable of treating infections caused by fungi, parasites and other agents susceptible to this antibiotic. The invention covers the stable formulation of amphotericin B in its superaggregate form in presence of sodium deoxycholate, freeze-dried (lyophilized) and sterile, uniting the requirements for an injectable formulation for use enteral, parenteral or topical, with reduced toxicity in comparison to the conventional forms that transmit amphotericin B.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 02/00157

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C07G 11/00, A61K 31/7048; 47/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C07G, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, PAJ, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gaboriau F. et al., "Physico-chemical properties of the heat-induced "superaggregates" of amphotericin B", Biophysical Chemistry 66 (1997) pages 1 December 2003 (01.12.03) *the whole document.*	1-11
X	Petit C. et al., "In-vivo therapeutic efficacy in experimental murine mycoses of a new formulation of deoxycholate-amphotericin B obtained by mild heating" *the whole document.*	1-11
A	US 5059591 A (JANOFF et al.) 22 October 1991 (22.10.91) *abstract; column 4, line 64-column 5, line 2.*	1-11
A	Jasek W., "Amphotericin B "BMS" 50 mg-Trockensubstandz zur lokalen und systemischen Anwendung", Austria Codex Fachinformation 2002/2003; ISBN 3 85200 150 1 *pages 286-288.*	1-11

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
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„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 April 2003 (15.04.2003)	1 July 2003 (01.07.2003)
Name and mailing adress of the ISA/AT Austrian Patent Office Dresdner Straße 87, A-1200 Vienna Facsimile No. 1/53424/535	Authorized officer KRENN M. Telephone No. 1/53424/435

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 02/00157

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Claim 1 should be devided in two claims, namely:
1., a process of obtaining colloidal dispersions
2., a composition based on superaggregates of amphotericin B

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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